PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 9/127

A1

(11) International Publication Number: **WO 99/65465**

(43) International Publication Date: 23 December 1999 (23.12.99)

(21) International Application Number: PCT/GB99/01911

(22) International Filing Date: 16 June 1999 (16.06.99)

(30) Priority Data:
9813100.6 18 June 1998 (18.06.98) GB

(71) Applicant *(for all designated States except US)*: THE SECRETARY OF STATE FOR DEFENCE [GB/GB]; Defence Evaluation and Research Agency, Ively Road, Farnborough, Hampshire GU14 0XL (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: GREGORIADIS, Gregory [GR/GB]; London School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX (GB). ZADI, Brahim [DZ/GB]; London School of Pharmacy, 29–39 Brunswick Square, London WC1N 1AX (GB). JAYASEKERA, Pramukh, Nalaka [GB/GB]; CBD Porton Down, Salisbury, Wiltshire SP4 0JQ (GB).

(74) Agent: BOWDERY, A., O.; D/IPR, Formalities Section, Poplar 2, MOD Abbey Wood # 19, Bristol BS34 8JH (GB).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD OF FORMING LIPOSOMES

(57) Abstract

A method of preparing a composition of a reagent, which method comprises the steps of (i) forming empty liposomes; (ii) mixing the liposomes from step (i) with a sugar solution and a reagent; and (iii) drying the mixture from step (ii). The compositions will generally comprise less than 10 % w/v sucrose. The method can be used to form small liposomes with high entrapment efficiency. It is particularly useful in the production of pharmaceuticals.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Method of Forming Liposomes

The present invention relates to a method of forming liposomes, to liposomes obtained thereby and their use, in particular in pharmaceutical applications.

The use of liposomes is well known in a wide variety of fields, including the pharmaceutical and cosmetic fields, where they are used as carriers for drugs and other reagents which are suitable for application to the skin.

Various methods are known for preparing liposomes. For example, they may be prepared by a dehydration/rehydration technique in which a lipid is dissolved in an organic solvent such as chloroform, dichloromethane or an alcohol such as methanol or ethanol. The solution is then dried for example using a rotary evaporator, in order to form a film of lipid on the wall of the evaporator vessel. Addition of water or an aqueous solution such as a buffer to the dry film results in the formation of multilamellar liposomes. This forms a first step in the production of vesicles using various methods. Subsequent treatment may lead to dehydration/rehydration vesicles or DRVs (Kirby and Gregoriadis, Biotechnology (1984) 2, 979-984). Alternatively, subsequent treatment by sonication of lipid suspensions to form for example unilamellar liposomes (A.D. Bangham et al., J. Mol. Biol. 13, 238 (1965)).

Other methods, which are well documented in the art, include detergent removal (Y. Kagawa et al., J. Biol. Chem. (1971 246, 5477), reverse-phase evaporation (F. Szoka and D. Papahadjopoulos, Proc. Natl. Acad. Sci, USA (1978) 75, 4194) and ether injection (D. Deamer et al., Biochim. Biophys. Acta, (1976) 433, 629) as well as the freeze drying methods (see for example Ohsawa et al., Chem. Pharm. Bull, (1984) 32, 2442-5 and Kirby and Gregoriadis (1984) supra.) and freeze thawing

methods (D.D. Lasic "Liposomes: from Physics to Application, Elsevier, 1993, p98).

Different methods of preparation lead to liposomes of different sizes and other characteristics. Liposomes can be used to encapsulate materials such as biologically active materials such as pharmaceuticals including vaccines, as well as non-pharmaceutical agents such as materials which affect skin, such as artificial tanning preparations and other beauty aids. Encapsulation techniques vary depending upon the nature of the reagent to be encapsulated and the size and characteristics of the generated liposome.

The size of liposomes is important in terms of their application. In some instances, large liposomes may be required, for example, where particulates including microorganisms such as bacteria are to be encapsulated for example for vaccine use as described in WO 95/09619.

Small liposomes however are preferable for many applications. This is because small liposomes are removed by the reticulo-endothelial system (RES) less rapidly and to a lower extent compared to large liposomes (over 200nm in size). The uptake by the RES increases with the size of the vesicles. Furthermore large liposomes injected intramuscularly are unable to reach the regional lymph nodes with good efficiency and to deliver vaccines and other agents to these sites (Gregoriadis G. Liposomes as Drug Carriers: Recent Trends and Progress, Wiley Chichester 1988).

Liposome formulations of various drugs can be optimized in terms of drug content, stability, biodistribution patterns and cellular uptake by changing physicochemical parameters of liposomes such as phase transition temperature, size, size distribution, surface charge, surface hydration with compounds bearing hydrophilic groups and size distribution.

Liposome size is a parameter which determines the fraction cleared by the RES (Senior et al. Biochem., Biophys, Acta (1985) 839, 1-8; Nagayasu et al., Biol. Pharm. Bull. (1995) 18(7), 1020-1023. Small liposomes can be prepared by the use of high pressure homogenizers (Talsma et al. Drug Development and Industrial Pharmacy (1989) 15(2) 197-207, Vemuri S et al. Drug Development and Industrial Pharmacy (1990) 16(15), 2243-2256) but large amount of lipids have been used in order to achieve an acceptable entrapped drug to lipid mass ratio. In another approach (Gregoriadis et al., Int. J. Pharm. 65 (1990) 235-242), the microfluidization of multilamellar dehydration-rehydration vesicles (DRVs) in the presence of unencapsulated drug produced vesicles with sizes less than 200nm., retaining quantites of the originally entrapped solute.

The vesicle stabilization effect of adding sugar after preparation of liposomes has been established (Crowe L.M. et al. Arch. Biochem. Biophys. 242 (1985) 240-247, Hauser et al. Biochem. Biophys. Acta (1987) 897, 331-334), for instance when drug containing liposomes are freeze dried for storage and then rehydrated for use.

The applicants have found an improved way of preparing liposomes and particularly small liposomes, which reduces the number of preparation steps and forms stable liposomes, with high entrapment efficiency.

According to the present invention there is provided a method of producing a liposome preparation of a reagent, which method comprises the steps of

(i) forming empty liposomes;

(ii) mixing the liposomes from step (i) with a sugar solution and said reagent; and

(iii) drying the mixture from step (ii).

On rehydration of the dried material from step (iii), liposomes, encapsulating the reagent are formed. The

increase in size of the liposomes thus obtained as compared to the liposomes obtained in step (i) is much lower when compared to the liposomes in preparations which do not include a sugar. The need for further extrusion, microfluidisation or homogenisation steps as outlined above may thus avoided.

It is established that during drying in the presence of appropriate concentrations of sugars, fusion and aggregation of liposomes is prevented to a certain extent by the formation of an amorphous glass (Crowe et al Arch. Biochem. Biophys. 242 (1985) 240-247) and interaction of sugar with the phospholipid headgroups (Crowe et al. Cryobiology 31 (1994) 355-366). In early studies dehydration/rehydration vesicles (DRV's) were performed without using sugars as stabilizers, the procedure being based on induction of fusion/ aggregation of performed small unilamellar vesicles upon controlled rehydration (Kirby Gregoriadis, 1984). On this basis, one could predict that the total stabilization of small unilamellar vesicles by the presence of appropriate amounts of sugars will lead upon reconstitution to the original SUV's to a very low entrapment.

This has unexpectedly not found to be the case. Although as with all liposomes, the degree of entrapment of reagent depends to some extent on the ratio lipid:reagent in the system, the amount of reagent which is encapsulated within the liposomes obtained using the method of the invention is expected to be good.

Furthermore, physical and chemical stability of liposomes is required for their application as a drug delivery system. Liposomes in the state of aqueous dispersions are subjected to hydrolysis and physical changes during storage including leakage of encapsulated drugs, and changes in vesicle size due to aggregation or fusion. The physical and chemical stability of liposomes produced by the method of the invention is expected to be good.

Thus this method gives rise to the possibility of obtaining small highly loaded vesicles or liposomes, which, as outlined above, may be particularly useful in the formation of pharmaceutical compositions. Thus this method may be used to prepare encapsulated materials of many types.

It is particularly suitable however for the production of small liposomes for pharmaceutical use. In this case, the reagents used in the method will comprise a biologically active material such as a pharmaceutical or drug. For this purpose, the liposomes obtained in step (i) are suitably small unilamellar vesicles with an average size, for example in the range of from 25nm to 90nm, preferably in the range of from 50 to 90nm and conveniently from 70 to 90nm. Liposomes obtained ultimately from the process of the invention will still be small, with average size of less than 500nm, usually from 100-200nm.

The liposomes used in step (i) are empty liposomes, obtained by any of the conventional methods, for example using a classical method as described above. Any liposomes which are produced which have an average size which is too large for the desired purpose, may be reduced for example using sonication, homogenisation, extrusion or microfluidisation techniques as are known in the art.

Lipids used in the production of the liposomes are well known in the art. They include for example, lecithins such as phosphatidylcholine (PC), dipalmitoyl phosphatidylcholine (DPPC), distearoyl phosphatidylcholine (DSPC) or charged lipids in particular anionic lipids such as phosphatidic acid or cationic lipids such as stearylamine, optionally in the presence of cholesterol. A further preferred lipid is DSPC. The selection of lipid will depend, to some extent on the nature of the active agent and the intended purpose of the liposome.

Suitable sugar solutions for use in step (ii) include aqueous solutions of monosaccharides such as glucose and fructose, disaccharides such as lactose or sucrose as well as polysaccharides. A particularly preferred sugar for use in the method of the invention is a disaccharide such as sucrose or lactose or a monosaccharide such as glucose. In particular, the sugar is sucrose.

Suitably the amount of sugar used in step (ii) is such that the mass ratio of sugar to lipid is in the range of from 1:1 to 6:1 w/w, suitably from 1:1 to 5:1 w/w. It has been found that the greater the amount of sugar present, the lower the increase in size of the liposomes obtained following rehydration as compared to those obtained in step (i). However, the degree of entrapment of the reagent maybe lower. Thus the precise selection of ratios used will depend upon the required end use, with a balance being determined between the degree of entrapment for a given lipid content and liposome size. The difference this makes to the liposome formation varies to a certain extent, depending upon the particular reagent employed as discussed further below. Suitably, the amount of sugar present is less than 10%w/v of the composition.

It has further been found that increasing the volume of the sugar solution used in the process, by reducing the concentration of the sugar solution, may enhance entrapment. Suitable concentrations of sugar solutions are from 20 to 200mM, preferably from 30 to 150mM.

In addition, it has been found that if the subsequent rehydration is effected at elevated temperatures, for example of from 30 to 80°C, in particular from 40 to 65°C and especially at about 60°C, entrapment values can be increased. This has been found to be effective with liposomes comprising PC and CHOL, which would usually be formed at room

temperature. There may be some size increase as compared to the starting liposomes when using elevated temperatures in this way, and therefore, this should be taken into account in selecting the particular conditions used to produce liposomes in any particular case.

Other factors which have been found to affect entrapment include the particular nature of the reagent, such as the drug, being encapsulated and in particular, its solubility, and the amount of reagent present. The solubility of the reagent, may in some instances, limit the amount that can be dissolved in step (ii) and thus entrapped in the liposome. Other factors which affect the amount of reagents which are entrapped include the interactions of the reagent with the lipids forming the liposome, and the permeability of the liposome to the reagent.

Where high concentrations of reagent are present in the solution used in step (ii) of the reaction, the percentage entrapment may be lower. Therefore, for reasons of economy, there may be an advantage in reducing the amount of reagent used.

The selection of conditions which will give liposomes of the desired size and loading, including the sugar: lipid mass ratio, the selection of lipid, the concentration of the sugar solution used, the amount of reagent included in the solution, and the temperature of rehydration, can be determined using routine methods for any particular reagent.

The drying step (iii) above may be carried out using conventional methods, for example by freeze drying, spray drying, flash crystallisation, air stream drying (for example on a fluidised bed), vaccuum drying, oven drying or any other method known in the art. Although the mechanical properties of the products of these two processes may be different, with the product of a spray drying process being a discrete and

frequently flowable powder, and freeze drying producing a solid cake, the properties of the liposomes on rehydration in terms of their stability and entrapment is broadly similar.

For many applications, including the production of pharmaceutical compositions, spray drying may be preferable as a result of the suitability of the mechanical properties of the product for further processing.

The product of freeze-drying comprises a block of porous cake which has relatively poor mechanical properties. The use of jet milling of the cake to achieve better properties can be effected, but damage can occur in this additional step.

Spray drying can achieve a dry product with good mechanical properties that can be delivered by inhalation, or reconstituted in water and administered by the parenteral route.

The subsequent rehydration step may be carried out during the manufacture process or alternatively, the composition may be supplied in the dry state and rehydrated at the site of intended use, for example in the hospital or pharmacy where an encapsulated pharmaceutical is to be administered to patients.

The liposomes obtained have a good stability resulting in a long shelf life of the product. This is important for example for cosmetics, toiletries and pharmaceuticals.

As discussed above, the method is particularly suitable in the preparation of relatively small liposomes with a high loading of reagent. This is particularly desirable for pharmaceutical applications such as the delivery of materials such as polymeric or protein drugs, DNA vaccines, gene therapy vectors or chemicals. Suitable chemicals include antibiotics such as oxytetraclines, β-lactam antibiotics such as penicillins such as pencillin G, ampicillin or amoxycillin, or cephalosporins,

anticancer drugs, hormones, immunotherapy agents, antiviral agents, anti-inflammatory compounds etc.

Liposome products obtained using the above described method may be formulated as pharmaceutical compositions, for example by combining them with pharmaceutically acceptable carriers or excipients. The formulations may be suitable for oral, parenteral in particular intravenous, or topical application, for example to the skin or to mucosal surfaces. A particular useful composition of the invention is a composition which is suitable for application by aerosol or inhaler. For this purpose, it has been found that high phase transition neutral lipid-based liposomes such as those formed from mixtures of DSPC and cholesterol are suitable. When produced in accordance with the invention, extrusion prior to drying may not be necessary.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings in which:

Figure 1 is a graph showing the size evolution of dipalmitoyl phosphatidylcholine (DPPC) and cholesterol (CHOL) liposomes from sonication to freeze drying in the presence of 0.0357M sucrose, and rehydration;

Figure 2 is a graph illustrating the effect in the method of the invention of the molarity of sucrose on the PC:CHOL liposomes entrapping FITC-Albumin, on the size distribution (% distribution: intensity) obtained after freeze-drying and rehydration;

Figure 3 is a graph showing the effect on the method of the invention of sucrose molarity on the size distribution after rehydration of PC:CHOL liposomes entrapping epidermal growth factor (EGF);

Figure 4 is a graph showing a comparison of the size distribution of extruded and rehydrated PC:CHOL liposomes produced according to the invention, entrapping FITC-albumin;

Figure 5 is a graph showing the size distribution of extruded and freeze-dried liposomes obtained by the method of the invention encapsulating carboxyfluorescein (CF); and

Figure 6 is a graph showing the size distribution of various liposome compositions of the invention.

In the following examples, egg phophatidylcholine (PC), dipalmitoyl phosphatidylcholine (DPPC) and distearoyl phosphatidincholine (DSPC) were purchased from Lipoid GmbH, Ludwigshafen, Germany, cholesterol, carboxyfluorescein (CF), fluorescein isothiocyanate labelled albumin (FITC-albumin), riboflavin, daunorubicin, doxorubicin, Triton X-100, sucrose, glucose and sodium dodecylsulfate (SDS) from Sigma London. Epidermal growth factor (EGF) was a gift from the Centre of Biological Sciences Havana, Cuba. $Na^{125}I$, C^{14}-labelled hydroxypropyl-β-cyclodextrin, ^{14}C-labelled penicillin were purchased from Amersham International (Amersham, UK). Labelling EGF with ^{125}I was done according to the chloramine T method. All other reagents were of analytical grade.

Example 1

Freeze Drying Method

Solute-containing DRV liposomes were prepared as follows: Various lipid mixtures, in particular mixtures of PC:CHOL and DPPC:CHOL in a molar ratio of 1:1 were dissolved in chloroform. Following evaporation of the solvent in a rotary evaporator at 37°C, a film was formed on the wall of a round-bottomed spherical flask. Multilamellar vesicles (MLV) were generated by dispersing the lipid film at temperatures in excess of the lipid transition temperature (>Tc) (which was in some cases room temperature) with double distilled water. The suspension was adequately sonicated to produce small

unilamellar vesicles (SUV) which were centrifuged to remove the metallic particles.

The SUV suspension was then transferred in a vial in which the desired amount of a selected drug (either FITC-Albumin (1mg), CF (1mg), hydroxypropyl-β-cyclodextrin (2mg) or EGF (150μg)) in solution was added as well as 0.0357M sucrose, and water added so as to attain the desired molarity of sucrose.

The preparation was then frozen and then freeze-dried over a sufficient time (according to the final volume). The dry cake was then subjected to controlled re-hydration at a temperature >Tc (e.g. 60°C) for 15 mins. by adding 100μl of distilled water. The preparation was diluted in PBS to have a specific gravity allowing the separation of the free drug from liposomes by ultracentrifugation.

Liposome size after rehydration was determined by photon correlation spectroscopy using an Autosizer 2C-Malvern (Malvern Instruments UK), equipped with a 25mw helium/neon laser. Mean diameter and size distribution were obtained.

Z average mean diameters, polydispersity index cumulative and differential distribution were recorded as function of the sucrose molarity or where the DRVs were extruded, according to their size. For the preparations exhibiting large size (up to 6 microns), a Mastersizer (Malvern) was used.

Entrapment values for the drugs were determined after ultracentrifugation of liposomes at 40,000xg. The amount of encapsulated material was calculated as percent of total CF, FITC-albumin, EGF or hydroxypropyl-β-cyclodextrin used.

Total and encapsulated amount of carboxyfluorescein and FITC-albumin were measured by fluorescence photometry at λ emission = 486 nm and λ excitation = 514 nm for CF and λ emission = 495

nm and λ excitation = 520 nm for FITC-albumin from the pellet dissolved with Triton or SDS (5% final concentration). The carbon 14 emission from labelled hydroxypropyl-β-cyclodextrin was measured by assay of radioactivity in a β scintillation counter.

The results are shown in Table 1:

Table 1

Entrapped material	Sucrose Molarity	Size after rehydration ± (SD) nm	% entrapment ± (SD)
FITC-Albumin *	40 mM 65 mM 135mM without sucrose	286.9 (29.2) 265.4 (6) 254.8 (6.5) 5250 (25)	87.5 (0.5) 70.2 (8.5) 52.7 (2.3) 84.2 (2.7)
Carboxy-fluorescein #	35.7 mM 71.4 mM 135 mM without sucrose	163.8 (25) 124.9 (2) 129.35 (1) 6200(40)	31.5 (0.1) 30.75 (0.05) 30.7 (0.05) 55.45 (0.05)
EGF# EGF ** EGF # EGF# EGF⊕ EGF⊕	35.7 mM 64.6 mM 126 mM without sucrose 35.7 mM without sucrose	144.8(32) 167.4(6.8) 146.4(1.3) 1276.7(100.7) 127.6(2.8) 2495.7(329)	33.5(6.9) 42(1.5) 29.6(1) 22.3(2.10) 25.5(0.2) 31.9(0.85)
Hydroxyprop-yl-β-cyclodextrin #	40mm without sucrose	133 10,000	26 24.4

where * indicates a liposome formed from a mixture of 32:32 μmoles PC:CHOL;

= 16:16 μmoles PC:CHOL;

** + 64:64 μmoles PC:CHOL; and

⊕ = 16:16 μmoles DPPC:CHOL

The results show that at a moderate degree of stabilization by sucrose, reconstitution allowing a certain extent of fusion (Fig 1) can lead to a quite high percentage entrapment.

Although similar percentage encapsulation of FITC-Albumin was achieved in the presence or absence of 40 mM of sucrose, (87% and 84% respectively) the final size was much smaller for the preparation where sucrose was used.

By varying the molarity of sucrose in different preparations of FITC-albumin containing-liposomes (Table 1), different size distributions of the liposomes could be obtained (Fig 2) although entrapment values decreased with increasing molarity.

Encapsulation of EGF and CF was performed in the presence of sucrose at different molarities. The percentage entrapment values were equal to those obtained with other preparations using the same amount of lipids but performed in the absence of sucrose. (c.f. Table 1) In this case, the molarity of sucrose did not affect the percentage entrapment values but did impact on the sizes and size distribution (Fig 3).

The Z average diameters of DRV liposomes produced in the presence or absence of sucrose are presented in Table 1. Results show that smaller vesicle size is achieved when sucrose is used at high molarity corresponding to narrower size distribution and so to moderate percentage entrapment values. The values of percentage entrapment are proportional to the size and size distribution width.

At two different molarities of sucrose we can measure almost the same two average diameters of two populations of vesicles which have got different widths (Figure 3).

For liposomes entrapping EGF prepared at 35.70 mM and 126 mM of sucrose the two average diameters after rehydration were 144.8 ±32nm and 146.4 ±1.3nm respectively.

If we look at the size distribution we observe that it is narrower for the 126 mM sucrose preparation. Decreasing the

size of liposomes and narrowing the size distribution by using high molarity of sucrose (over 36 mM) does not induce low entrapment values. Using two different concentrations of sucrose (35.7mM and 135mM), it was found that liposomes entrapping the same amount of CF (around 30%) with narrower size distribution (PDI=0.13) could be prepared than with liposomes prepared at 135 mM sucrose.

Example 2

Comparison of Liposomes of the Invention with Extruded Liposomes

In order to compare the method of the invention with that of extrusion, which also leads to vesicle size reduction, we used an extruder to treat DRV liposomes prepared without using sucrose. Liposomes prepared as described in Example 1 was compared to those obtained by an extrusion process.

DRV liposomes, prepared without sucrose were subjected to extrusion using a high pressure filter holder. Liposomes before the elimination of non-entrapped solute, were passed through polycarbonate membranes whose pore size were 1.2μm., 0.4μm., 0.2μm. and 0.1μm. At each extrusion step, five passes through the same membranes were accomplished.

The free solute was then separated from the extruded vesicles by ultracentrifugation. The pellet was suspended in 1 ml of PBS (PH=7.4).

The size of the liposomes were then measured as described in Example 1. The size distribution was compared with that of similar liposomes obtained as described in Example 1. The results are shown in Figures 4 and 5. It was found that the extruded liposomes demonstrated a narrower distribution of vesicle sizes.

The entrapment of material within the comparative liposomes was measured both before and after extrusion. The results are shown in Table 2.

Table 2

Membrane pore size (nm)	reagent material	size before extrusion ±(SD)	size after extrusion ±(SD)	final % entrapment ±(SD)
200	FITC-albumin	5250 (25)	210.75 (4)	29 (2)
200	CF	6300 (150)	213.9 (0.7)	6.3 (1)
100	CF	6220 (130)	158.7 (0.3)	6 (0.5)
400	EGF	2495 (329)	327.4 (12.68)	9.15 (0.35)
200	EGF	3128 (763)	220.7 (4.12)	6.35 (1.25)

The average diameters and entrapment values are presented in Table 2. They show that low entrapment values and narrow size distribution (PDI≤0.1) are obtained for extruded liposomes. The low entrapment values combined with the requirement for an additional step (extrusion) for the preparation of small sized liposomes significant reduces the practical application of this method.

Fig 5 shows an overlaid size distribution of extruded liposomes entrapping CF (6% entrapment) and of freeze-dried liposomes in presence of 135 mM sucrose with 30% of CF encapsulated.

The narrow distribution of vesicle size obtained with extrusion is not of prime importance since the corresponding percentage entrapment values are poor.

Example 3
Size Distribution of Liposomes of the Invention

The use of phospholipids with a high phase transition temperature can allow the improvement of this technique concerning size distribution width. This was achieved when equimolar DPPC:CHOL liposomes entrapping EGF were formulated as described in Example 1 in the presence of 35.71 mM sucrose. EGF entrapment values were 25%. However, liposomes exhibited a narrower size distribution (Z average = 128 nm) than the corresponding PC:CHOL preparation. The results are shown in Figure 6. Thus it appears that, in this case, selection of lipids with high phase transition temperatures is preferred in order to achieve liposomes with a narrow size distribution.

Example 4
High Yield Entrapment of Riboflavin into Small Liposomes

Equimolar phosphatidylcholine (390μmoles) and cholesterol were used to prepare small unilamellar vesicles (SUV) by sonication. SUV were then mixed with riboflavin (12 mg) and increasing amounts of sucrose (0-5 mg per mg of total lipid). The mixtures were spray dried and then rehydrated. Drug entrapment was measured in the suspended pellets of the centrifuged preparations. The size of SUV in the final vesicle preparations was measured by photocorrelation spectroscopy or in a Mastersizer. Results are shown in Table 3.

Table 3

SUV z average mean size (nm±SD)	Amount of sucrose/amount of lipid	Entrapment (% of drug used)	Vesicle z average mean size (nm±SD)
78.1±0.5	0	45.8%	4690
77.0±0.4	1	47.5%	313.2±1.5
67.0±0.1	3	18.8%	155.4±1.5
80.5±0.9	5	11.0%	106.8±1.5

It appears that spray-drying of small liposomes (SUV) in the presence of drug and sucrose (1mg/1mg lipid) leads to relatively small liposomes entrapping nearly half (47.5%) of the amount of drug used. By increasing the amount of sucrose present, vesicle size is reduced further with, however, a concomitant reduction of entrapment values.

Example 5

Liposomes containing glucose

The procedure of Example 1 was repeated but using riboflavin as the active agent in an amount to give a concentration in the solution of 1mg (total in 1ml) and, in some instances, using glucose in place of sucrose. Liposome size on rehydration was measured as described in Example 1. The riboflavin encapsulation efficiency was calculated by measuring total and encapsulated riboflavin by fluorescence photometry at emission wavelength =480nm and excitation wavelength =520nm. The results are shown in Tables 4 and 5.

Table 4

Sucrose/lipid Mass ratio	Size (nm)	Glucose/lipid Mass ratio	Size (nm)
0/1	1243.4	0/1	5210
1/1	591.8	1/1	908
3/1	168.9	3/1	306.8
5/1	144.9	5/1	267

Table 5

Sucrose/lipid Mass ratio	% entrapment	Glucose/lipid Mass ratio	% entrapment
0/1	59.3	0	45.87
1/1	78.0	1	52.97
3/1	47.83	3	45.21
5/1	34.81	5	39.4

In terms of quality, adding glucose to the SUV liposomes instead of sucrose produced the same stabilisation effect. The entrapment values of riboflavin were of a similar order in both lipid to sugar ratios of 3g/g and 5g/g of glucose or sucrose. (Table 5).

Liposomes prepared by adding the equivalent amount of glucose exhibited larger vesicles size upon rehydration compared to the samples prepared in presence of sucrose (Table 4).

Example 6

Effect of rehydration temperature on liposome formation

The method of Example 1 was repeated using equimolar PC:CHOL liposomes, a sucrose solution (68.7mM) and 5mg ^{14}C penicillin (Pen G)as the active agent. In this case however, the rehydration was carried out at various temperatures. Specifically, some preparations were rehydrated at room temperature while others were heated at 60°C for 15 min.

Entrapment efficiency was determined after ultracentrifugation of the prepared liposomes at 40,000g, and then the radioactivity of the ^{14}C-penicillin was expressed as a percentage of the total amount in the supernatant and the pellet. The results are shown in Table 6.

Table 6

EPC: CHOL	Rehydration @ 25°C			EPC: CHOL	Rehydration @ 60°C		
Sucrose mol=68.7mM				Sucrose mol=68.7mM			
SUV size=68nm				SUV size=85.8nm			
Pen G	5mg			Pen G	5mg		
Suc/lip	Size(nm)	%entr	PDI				
0/1	5605	38.5	-				
	4920	45.5	-	Suc/lip	Size(nm)	%entr	PDI
AV	5262	42	-				
SD	342.5	3.5	-				
1g/g	95.7		0.38				
	69.7		0.38	1g/g	973		0.12
	95.7		0.38		926.4		1.0
AV	96	12.9	0.38	AV	949.7	34.4	0.56
SD	0.47		00	SD	23.3		0.44
1g/g	104		0.33				
	102.9		0.4	1g/g	1135		0.33
	101.5		0.43		1063.4		0.40
AV	102.8	14.1	0.39	AV	1099	40	0.36
SD	1.02		0.04	SD	35.8		0.03
3g/g	89.5		0.24				
	92.8		0.22	3g/g	232		0.31
	90.4		0.21		229		0.29
AV	90.9	6.2	0.22	AV	230.5	24.1	0.3
SD	1.39		0.01	SD	1.45		0.01
3g/g	93.7		0.2				
	88.8		0.43	3g/g	247.7		0.29
	84.5		0.53		244.6		0.27
AV	89	6.1	0.39	AV	246.15	23.6	0.28
SD	3.76		0.14	SD	1.55		0.01
5g/g	88.8		0.19				
	92.1		0.14	5g/g	271.6		0.30
	90.9		0.16		276.2		0.21
AV	90.6	6.3	0.16	AV	273.9	17.2	0.25
SD	1.36		0.02	SD	2.3		0.04
5g/g	89		0.16				
	88.7		0.12	5g/g	268.3		0.37
	86.9		0.15		266.3		0.27
AV	88.2	6.9	0.14	AV	267.3	17.9	0.32
SD	0.93		0.02	SD	1		0.05

In the above table, as well as the following tables, "AV" represents the average liposome size, "SD" is the standard deviation and "PDI" represents size distribution or polydispersity index.

Liposomes which were rehydrated at room temperature showed only a slight increase in size from 68nm (sonicated SUV) to only an average of 90nm but allowed an encapsulation of an average of 6.5% of the originally added penicillin. Even at high concentrations of sucrose, a degree of encapsulation could be achieved. The 100nm vesicles exhibit a percentage encapsulation of 14%.

Heating similar preparations during the rehydration step led to larger vesicles. Liposomes of an average diameter of 230nm could encapsulate 24% of the originally added ^{14}C penicillin (ratio of 3g sucrose/g of lipid). Increasing the amount of sucrose by increasing the mass ratio of sugar/lipid from 3 to 5 led to slightly larger vesicles exhibiting a lower percentage encapsulation.

Example 7

Encapsulation of ^{14}C penicillin in various sucrose concentrations

The method of Example 6 was repeated using equimolar PC:CHOL liposomes and ^{14}C penicillin (5mg) as the active agent but with either a high molarity sucrose solution (68.78 mM) or a more dilute sucrose solution (35mM).

The results are shown in Table 7, side by side with the 25°C rehydration results previously shown in Table 6.

Table 7

EPC:CHOL	Rehydration @ 25°C			EPC:CHOL	Rehydration @ 25°C		
Sucrose mol=68.7mM, SUV size=68nm				Sucrose mol=35mM, SUV size=83.5nm			
Pen G	5mg			Pen G	5mg		
Suc/lip	Size(nm)	%entr	PDI				
0/1	5605	38.5	-				
	4920	45.5	-				
AV	5262.5	42	-				
SD	342.5	3.5	-	Suc/lip	Size(nm)	%entr	PDI
1g/g	95.7		0.38				
	69.7		0.38	1g/g	211.6		0.3
	95.7		0.38		213.5		0.34
AV	96	12.9	0.38	AV	212.55	29.1	0.32
SD	0.47		00	SD	0.95		0.02
1g/g	104		0.33				
	102.9		0.4	1g/g	212.2		0.25
	101.5		0.43		215.3		0.27
AV	102.8	14.1	0.39	AV	213.75	31.8	0.26
SD	1.02		0.04	SD	1.55		0.01
3g/g	89.5		0.24				
	92.8		0.22	3g/g	200.8		0.06
	90.4		0.21		198.8		0.09
AV	90.9	6.2	0.22	AV	199.8	19.9	0.075
SD	1.39		0.01	SD	1		0.015
3g/g	93.7		0.2				
	88.8		0.43	3g/g	190		0.2
	84.5		0.53		192.3		0.16
AV	89	6.1	0.39	AV	191.15	19.0	0.18
SD	3.76		0.14	SD	1.15		0.02
5g/g	88.8		0.19				
	92.1		0.14	5g/g	198.7		0.04
	90.9		0.16		200		0.1
AV	90.6	6.3	0.16	AV	199.35	16.2	0.07
SD	1.36		0.02	SD	0.65		0.03
5g/g	89		0.16				
	88.7		0.12	5g/g	195.2		0.08
	86.9		0.15		196		0.06
AV	88.2	6.9	0.14	AV	195.6	14.8	0.07
SD	0.93		0.02	SD	0.4		0.01

The liposomes produced using a low molarity sugar solution exhibited an average size around 200nm, which was higher than those produced using high molarity sugar solution. However, the entrapment values were higher and polydispersity index lower.

It would appear therefore that decreasing the molarity of sucrose during liposome preparation is a better alternative to the use of high temperatures (c.f. Example 6) during the rehydration step in order to enhance entrapment. Although similar percentage encapsulation is achieved, the liposomes maintain smaller sizes when low molarity sucrose is used.

Example 8

DSPC Liposome Preparation

The method of Example 6 was repeated using DSPC and equimolar cholesterol to prepare liposomes. In this experiment, a high sucrose molarity (71mM) and penicillin (5mg) was used.

The results are shown in Table 8.

Table 8

DSPC: CHOL	Heated at 60°C for 15 min		
Sucrose molarity	71.16mM		
SUV size(nm)	77.2	PDI=0.27	
Mass sugar/lipid	Size(nm)	% Encapsulation	PDI
0/1	4645	50.35	-
	5005	51.43	-
AV	4825.0	50.9	-
SD	180.00	0.54	-
1	302		0.17
	282		0.34
	277.2		0.3
AV	287.1	41.9	0.3
SD	10.74		0.07
1	252.9		0.28
	241.3		0.25
	239.7		0.27
AV	244.6	37.7	0.27
SD	5.88		0.01
3	158		0.08
	152.3		0.18
	152.4		0.15
AV	154.2	18.8	0.14
SD	2.66		0.04
3	160.8		0.18
	158.8		0.14
	154.2		0.17
AV	157.9	19.3	0.16
SD	2.78		0.02
5	171.2		0.1
	166.9		0.14
	164.5		0.12
AV	167.5	13.9	0.12
SD	2.77		0.02
5	143.9		0.13
	142.4		0.11
	139.5		0.14
AV	141.9	15.3	0.13
SD	1.83		0.01

These results show that increasing the amount of sucrose resulted in decreased entrapment values of ^{14}C penicillin and decreasing average diameter. DSPC CHOL liposomes showed higher entrapment values and smaller sizes compared to PC:CHOL liposomes. This may be due to the high phase transition

temperature (Tc) of DSPC allowing higher stability upon heating.

Example 9

Doxorubicin encapsulation

Doxorubicin containing liposomes were prepared using the experimental conditions set out in Table 9.

Table 9

Experimental conditions

Doxorubicin (1.3 mg/ml)

Doxorubicin assay at excitation and emission wavelength of 480 nm and 560 nm respectively

Liposome Compositions

EPC:CHOL (38.2 mg total); vesicle size 68.2 nm

Doxorubicin used 0.5 mg

Sucrose used (mg)	0	38	114	190
Volume before freeze drying (ml)	1.51	1.51	4.5	7.5

DSPC:CHOL(40 mg total); vesicle size 59.7nm

Doxorubicin used: 0.5 mg

Sucrose used (mg)	0	40	120	200
Volume before freeze drying (ml)	1.58	1.58	4.73	7.9

The size of the rehydrated liposomes were determined as described in previous examples. Entrapment values for doxorubicin were determined after ultracentifugation as described above. Total and encapsulated doxorubicin were measured by fluorescence photometry at emission wavelength 490nm and excitation wavelength 560nm. The results are shown in Table 10.

Table 10

	Sucrose/lipid mass ratio	Entrapment	Size(nm)	PDI
	0/1	53	2276	0.99
EPC:CHOL	1/1	54.5	281.96	0.34
	3/1	47.1	133.5	0.15
	5/1	45.45	116.4	0.15
DSPC:CHOL	0/1	74	2373.8	1
	1/1	71.6	686.9	0.51
	3/1	67.95	179.53	0.28
	5/1	66.4	131.23	0.11

Doxorubicin was successfully encapsulated in small-sized liposomes. Equimolar PC:CHOL liposomes prepared with 5g sucrose to 1g of lipid exhibited a size of 116nm and an encapsulation efficiency of 45%. Increasing the sucrose to lipid ratio did not affect substantially the % encapsulation. Replacing PC with DSPC generated liposomes exhibiting higher percentage encapsulation.

Example 10

Effects of increasing sucrose concentration

The method of Example 4 was repeated using progressively higher concentrations of sucrose. The concentrations used together with the riboflavin entrapment figures and the liposome size results are shown in Table 11.

Table 11

The effect of sucrose concentration on the entrapment of riboflavin

SUV	Size (nm)	Sucrose (mM)	Sucrose/ lipid mass ratio (%w/v sugar)	Entrapment (%±SD)	Size nm ±SD)	PDI±SD
PC, CHOL	57.0	0.0	0.0	43.7	5200	
		40.0	1.0	78.0	591.8	
			3.0	47.8	168.9	
		292.1	10.0 (10.0%)	6.1±0.8	113.4±3.4	0.12±0.0
			15.0 (10.0%)	4.1±0.3	116.3±0.4	0.14±0.0

These results show that at high sucrose/lipid mass ratios, in particular in excess of 10%w/v sucrose, the entrapment figures are low, although the stabilisation effects on the size of the liposomes is good.

Example 11

Desoxyfructo-serotonin (DFS) encapsulation

Liposomes containing DFS were prepared using the conditions summarised in Table 12 below. The results including the entrapment figures and the size after rehydration is also shown in this table.

Table 12

Desoxyfruco-serotonin (DFS) (input 2 mg)				
Rehydrated at room temperature				
Sucrose molarity = 52 mM				
Lipid composition	Sucrose/lipid mass ratio	% Entrapment (±SD)	Size (nm) (±SD)	PDI (±SD)
EPC:CHOL	none	92.73 (0.23)	1152.9 (60.6)	1 (0)
	1	36.9 (0.42)	121.1 (7)	0.29 (0.01)
	5	12 (2.7)	134.5 (24)	0.15 (0.07)

In this example, good levels of entrapment were achieved as well as acceptable size stabilisation.

Claims

1. A method of preparing a composition of a reagent, which method comprises the steps of
(i) forming empty liposomes;
(ii) mixing the liposomes from step (i) with a sugar solution and a reagent; and
(iii) drying the mixture from step (ii).

2. A method according to claim 1 which further comprises the step of rehydrating the mixture from step (iii).

3. A method according to claim 1 or claim 2 wherein the liposomes formed in step (i) are small unilamellar vesicles.

4. A method according to claim 3 wherein the vesicles have an average size of from 25 to 100 nm.

5. A method according to claim 4 wherein the average size of the vesicles is from 50 to 100nm.

6. A method according to any one of the preceding claims wherein the composition comprises less than 10%w/v of sugar.

7. A method according to any one of the preceding claims wherein the concentration of the sugar solution used in step (ii) is from 20 to 200mM.

8. A method according to claim 7 wherein the concentration of the sugar solution is from 30 to 150mM.

9. A method according to any one of the preceding claims wherein the mass ratio of sugar to lipid used in step (ii) is from 1:1 to 6:1 w/w.

10. A method according to claim 9 wherein the mass ratio of the sugar to lipid used in step (ii) is from 1:1 to 5:1 w/w.

11. A method according to any one of the preceding claims wherein the sugar used in step (ii) is a dissacharide.

12. A method according to claims 11 wherein the sugar is sucrose.

13. A method according to any one of the preceding claims wherein the sugar used in step (ii) is a monossacharide.

14. A method according to claims 13 wherein the sugar is glucose.

15. A method according to any one of the preceding claims wherein step (iii) is effected by freeze-drying.

16. A method according to any one of claims 1 to 14 wherein step (iii) is effected by spray drying.

17. A method according to any one of claims 1 to 14 wherein step (iii) is effected by flash crystallisation.

18. A method according to any one of the preceding claims wherein the reagent is a biologically active material.

19. A method according to claim 18 wherein the biologically active material comprises a pharmaceutical.

20. A composition comprising a reagent, encapsulated by a method according to any one of the preceding claims.

21. A composition according to claim 20 which is a pharmaceutical composition.

22. A composition according to claim 20 or 21 which is in a form suitable for administration orally, topically, parenterally or by inhalation.

23. A method of administering a biologically active ingredient to a patient in need thereof, said method comprising administering to said patient a composition according to any one of claims 20 to 22.

Fig.1.

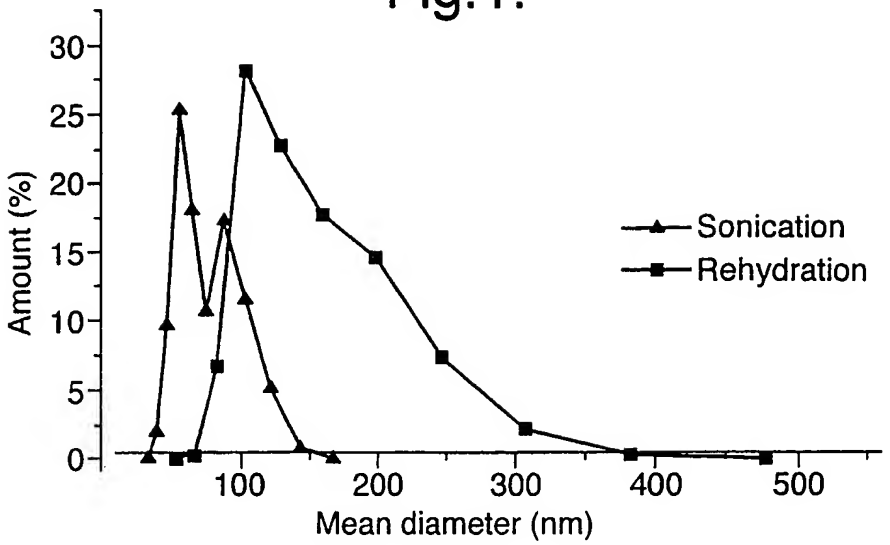

Amount (%)
30
25
20
15
10
5
0
100
200
300
400
500
Mean diameter (nm)
Sonication
Rehydration

Fig.2.

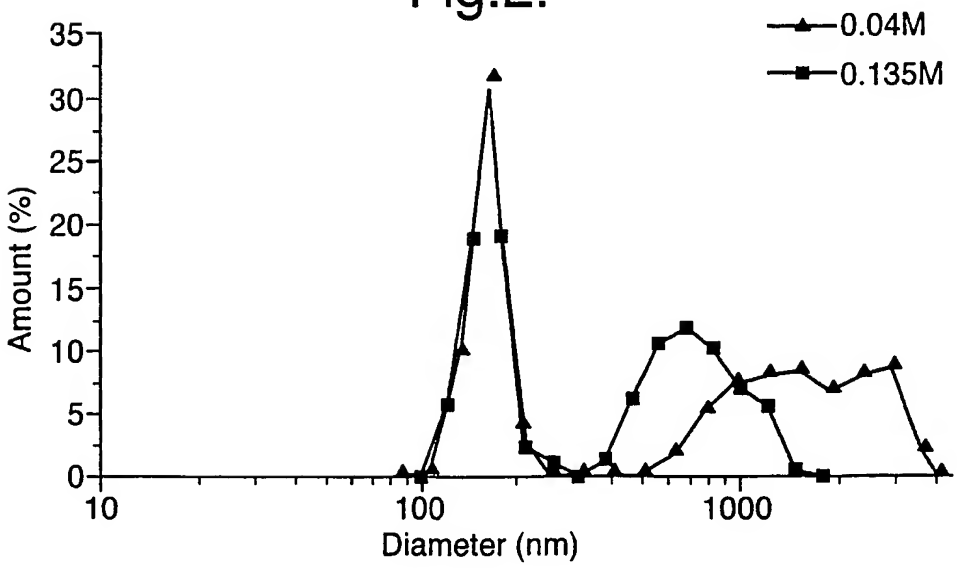

Amount (%)
35
30
25
20
15
10
5
0
10
100
1000
Diameter (nm)
0.04M
0.135M

Fig.3.

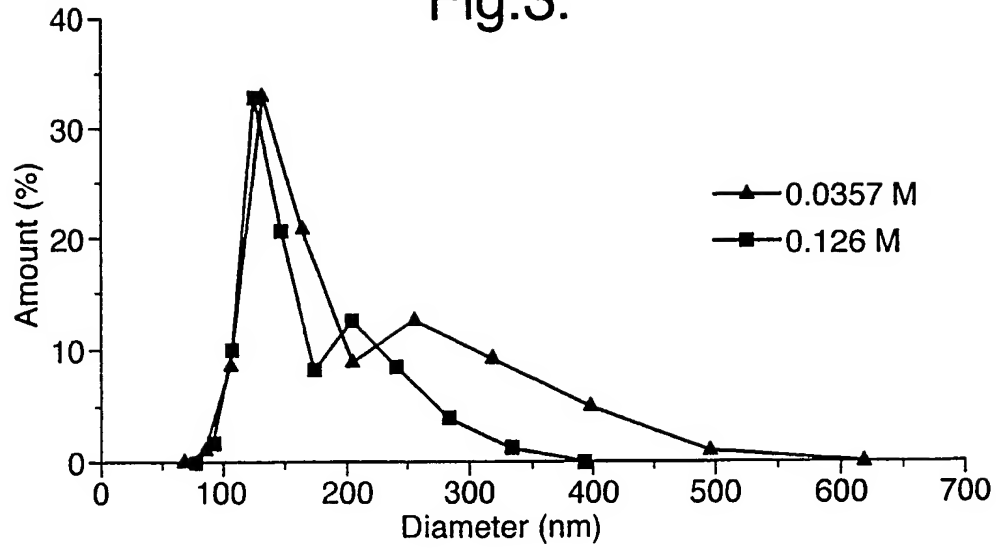

40
30
20
10
0
Amount (%)
0
100
200
300
400
500
600
700
Diameter (nm)
0.0357 M
0.126 M

Fig.4.

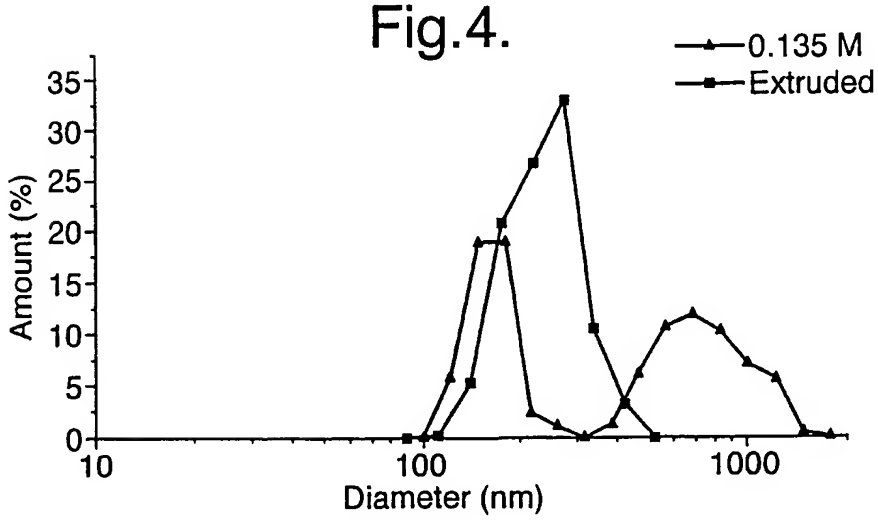

35
30
25
20
15
10
5
0
Amount (%)
10
100
1000
Diameter (nm)
0.135 M
Extruded

Fig.5.

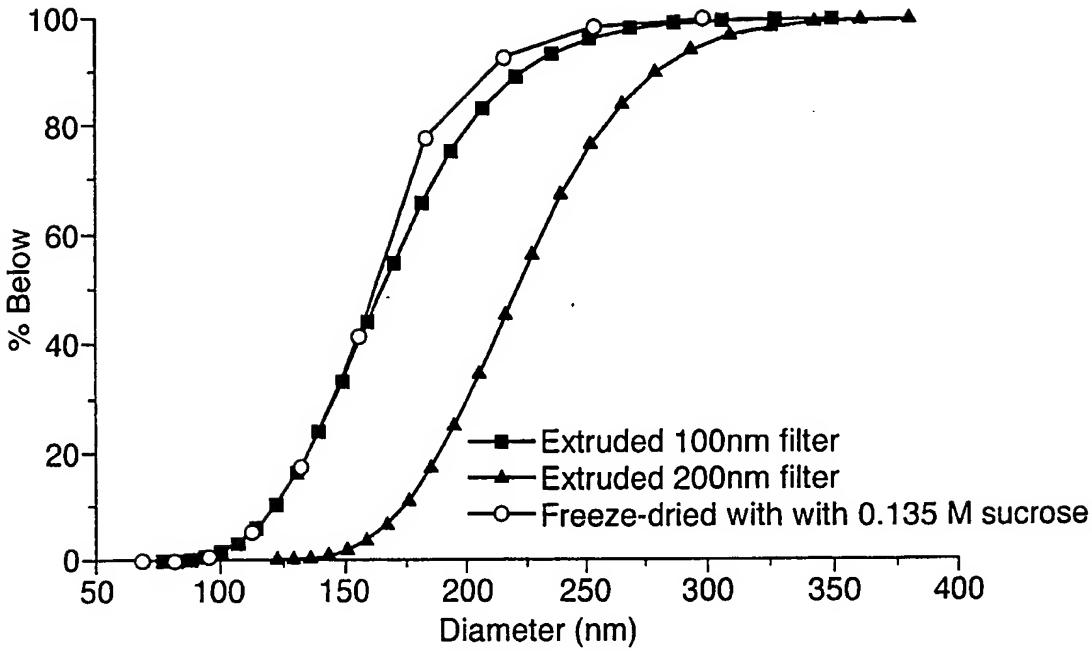
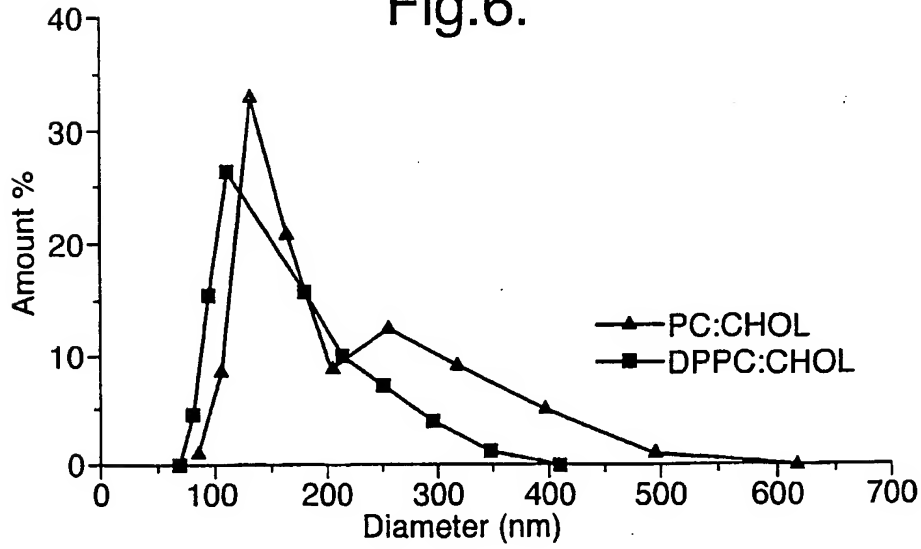

100
80
60
40
20
0
% Below
50
100
150
200
250
300
350
400
Diameter (nm)
Extruded 100nm filter
Extruded 200nm filter
Freeze-dried with with 0.135 M sucrose

Fig.6.


40
30
20
10
0
Amount %
0
100
200
300
400
500
600
700
Diameter (nm)
PC:CHOL
DPPC:CHOL

INTERNATIONAL SEARCH REPORT

Inte 'onal Application No
PCT/GB 99/01911

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ¹	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 35561 A (RUBINSTEIN ISRAEL ;UNIV PENNSYLVANIA (US); ONYUKSEL HAYAT (US)) 2 October 1997 (1997-10-02) page 7, line 13-25 page 28, line 15 -page 29, line 2 claims 1,2,10,18,19 ---	1-3,11, 15,19-23
P,X	ZADI, BRAHIM ET AL: "A novel method for high - yield entrapment of solutes into small liposomes" PROC. INT. SYMP. CONTROLLED RELEASE BIOACT. MATER. (1998), 25TH, 402-403 , XP002115605 the whole document --- -/--	1-3, 6-12,15, 18,20, 22,23

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

¹ Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 September 1999	30/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	La Gaetana, R

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inte· 'onal Application No
PCT/GB 99/01911

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 800 822 A (MITSUBISHI CHEM CORP) 15 October 1997 (1997-10-15) column 1, line 5-14 column 6, line 3-11 example 1	1
A	US 4 883 665 A (MIYAZIMA KOICHIRO ET AL) 28 November 1989 (1989-11-28) column 1, line 30-44 column 3, line 4-12 examples 2-4	1
A	WO 86 01103 A (LIPOSOME CO INC) 27 February 1986 (1986-02-27) page 2, line 23-26 page 3, line 19-34 page 7, line 6-9 page 8, line 18-26 page 9, line 8-16 claims 1-4,6	1

1

INTERNATIONAL SEARCH REPORT

national application No.
PCT/GB 99/01911

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 23 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inte ...tional Application No
PCT/GB 99/01911

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9735561 A	02-10-1997	AU 2426197 A	17-10-1997
		AU 2549297 A	17-10-1997
		CA 2250219 A	02-10-1997
		EP 0914094 A	12-05-1999
		WO 9735560 A	02-10-1997
EP 0800822 A	15-10-1997	CA 2202103 A	11-10-1997
		JP 10029930 A	03-02-1998
US 4883665 A	28-11-1989	CA 1256372 A	27-06-1989
		JP 6104622 B	21-12-1994
		JP 62030708 A	09-02-1987
WO 8601103 A	27-02-1986	US 4880635 A	14-11-1989
		AT 96311 T	15-11-1993
		AT 96310 T	15-11-1993
		AT 149347 T	15-03-1997
		AT 149346 T	15-03-1997
		CA 1270197 A	12-06-1990
		CA 1270198 A	12-06-1990
		CA 1283604 A	30-04-1991
		CA 1294548 A	21-01-1992
		CA 1305054 A	14-07-1992
		DE 3587639 D	02-12-1993
		DE 3587639 T	24-03-1994
		DE 3587640 D	02-12-1993
		DE 3587640 T	31-03-1994
		DE 3588146 D	10-04-1997
		DE 3588146 T	09-10-1997
		DE 3588148 D	10-04-1997
		DE 3588148 T	09-10-1997
		EP 0191824 A	27-08-1986
		EP 0190315 A	13-08-1986
		EP 0562641 A	29-09-1993
		EP 0561424 A	22-09-1993
		HK 1007494 A	16-04-1999
		HK 1007501 A	16-04-1999
		IE 68686 B	10-07-1996
		JP 2847065 B	13-01-1999
		JP 9020652 A	21-01-1997
		JP 2574999 B	22-01-1997
		JP 7145041 A	06-06-1995
		JP 2572553 B	16-01-1997
		JP 7165560 A	27-06-1995
		JP 2572554 B	16-01-1997
		JP 7145042 A	06-06-1995
		JP 2579441 B	05-02-1997
		JP 7145043 A	06-06-1995
		JP 2579442 B	05-02-1997
		JP 7145040 A	06-06-1995
		JP 7112968 B	06-12-1995
		JP 62500101 T	16-01-1987
		JP 8018973 B	28-02-1996
		JP 62500102 T	16-01-1987
		PT 80926 A,B	01-09-1985
		US 5578320 A	26-11-1996
		WO 8601102 A	27-02-1986
		US 5077056 A	31-12-1991

INTERNATIONAL SEARCH REPORT

...formation on patent family members

Inter onal Application No
PCT/GB 99/01911

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 8601103 A		US 5837279 A	17-11-1998
		US 5736155 A	07-04-1989
		US 5922350 A	13-07-1999
		CA 1329548 A	17-05-1994
		MX 9203291 A	01-08-1992